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Review

Application of Array-Based Comparative Genomic Hybridization to Clinical Diagnostics

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Microarray-based comparative genomic hybridization (array CGH) is a revolutionary platform that was recently adopted in the clinical laboratory. This technology was first developed as a research tool for the investigation of genomic alterations in cancer. It allows for a high-resolution evaluation of DNA copy number alterations associated with chromosome abnormalities. Array CGH is based on the use of differentially labeled test and reference genomic DNA samples that are simultaneously hybridized to DNA targets arrayed on a glass slide or other solid platform. In this review, we examine the technology and its transformation from a research tool into a maturing diagnostic instrument. We also evaluate the various approaches that have shaped the current platforms that are used for clinical applications. Finally, we discuss the advantages and shortcomings of "whole-genome" arrays and compare their diagnostic use to "targeted" arrays. Depending on their design, microarrays provide distinct advantages over conventional cytogenetic analysis because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. This new platform is poised to revolutionize modern cytogenetic diagnostics and to provide clinicians with a powerful tool to use in their increasingly sophisticated diagnostic capabilities. ***(J Mol Diagn 2006, 8:528–533; DOI: 10.2353/jmoldx.2006.060029)***

The application of microarray-based comparative genomic hybridization (array CGH) to diagnostics is transforming the field of clinical cytogenetics. Array CGH compares DNA content from two differentially labeled genomes. The two genomes, a test (or patient) and a reference (or control), are cohybridized onto a solid support (usually a glass microscope slide) on which cloned or synthesized DNA fragments have been immobilized (Figure 1). Arrays have been built with a variety of DNA substrates that may include oligonucleotides,[1] cDNAs,[2] or bacterial artificial chromosomes (BACs).[3] The resolution of the array is limited only by the size of the cloned DNA targets and the natural distance between these sequences located on the chromosome. The primary advantage of array CGH over fluorescence *in situ* hybridization (FISH) is the array's ability to detect DNA copy changes simultaneously at multiple loci in a genome. These changes may include deletions, duplications, or amplifications at any locus as long as that region is represented on the array. Thus, array CGH is a coordinated and concurrent FISH experiment over hundreds or thousands of loci. In contrast, FISH on metaphase or interphase cells is limited by the number of probes that can be used simultaneously.[4] In addition, FISH requires clinical suspicion that a specific locus in the genome has undergone copy-number change. This knowledge dictates the choice of probe for the FISH analysis and the examination of either interphase nuclei or metaphase chromosomes. Finally, FISH analysis on metaphase chromosomes detects only microdeletions,[4] since FISH—even on interphase nuclei—may fail to identify duplications.

There are many approaches to the design and development of microarrays for CGH applications. Some arrays have been designed to span the entire human genome.[5,6] These "whole-genome" microarrays often include clones that provide an extensive, albeit arbitrary coverage—for example, one clone every 1 mean bar (Mb), on average, across the genome.[6–8] Other arrays have contiguous coverage, within the limits of the genome.[5,9] These and other arrays have been constructed mostly for research applications and have proven their outstanding worth in gene discovery.[5,9] Whole-genome arrays are very valuable in screening the genome ("genome profiling") for DNA gains and losses at an unprec-

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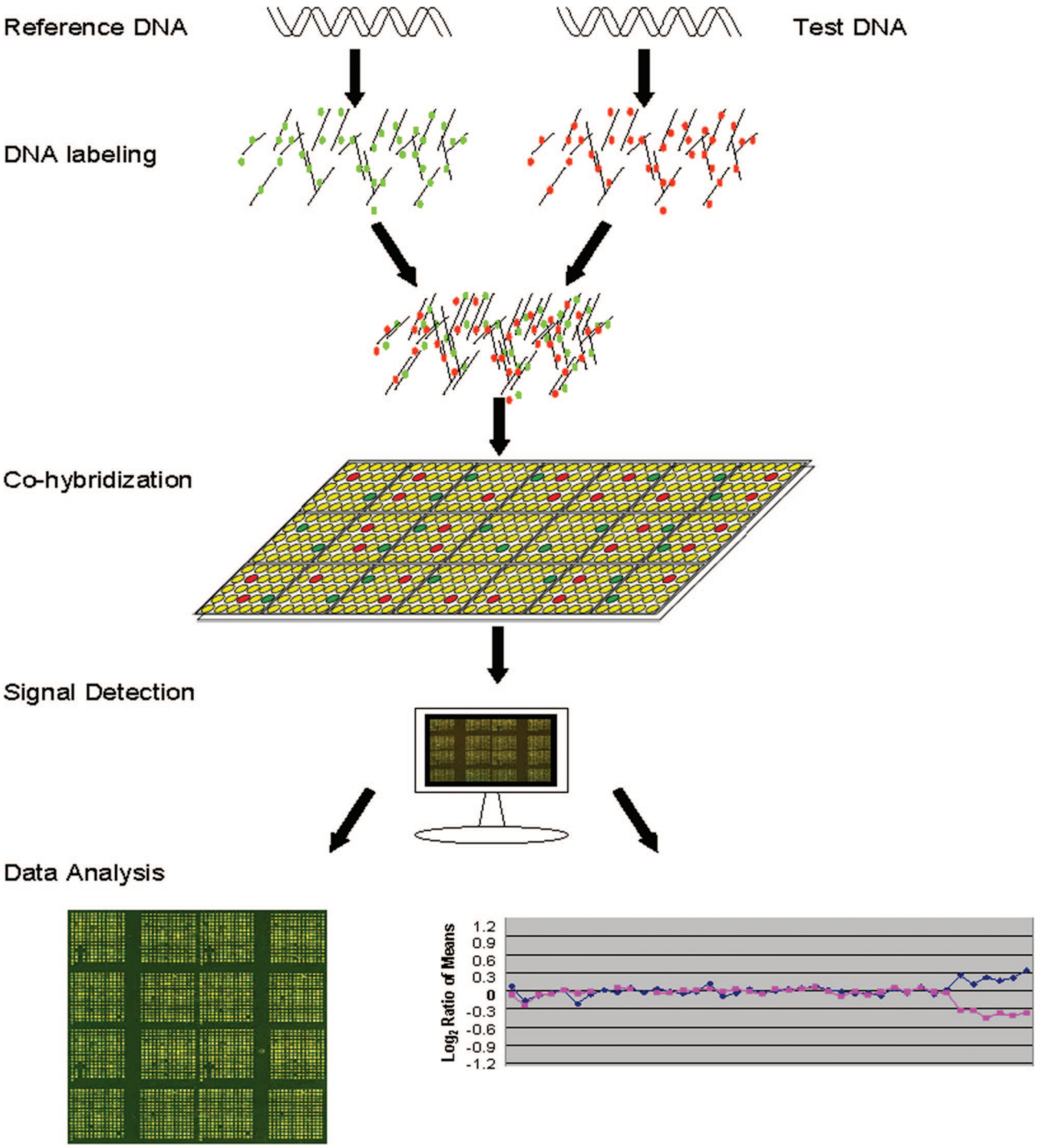

Figure 1. Schematic representation of CGH microarray technology. Whole genomic DNA from a control or reference (left) and genomic DNA from a test or patient (right) are differentially labeled with two different fluorophores. The two genomic DNA samples are competitively cohybridized with large-insert clone DNA targets that have been robotically printed onto the microarray (middle). Computer imaging programs assess the relative fluorescence levels of each DNA for each target on the array (lower left). The ratio between control and test DNA for each clone can be linearly plotted using data analysis software to visualize dosage variations (lower right), indicated by a deviation from the normal $\log_2$ ratio of zero.

edented resolution. The value of their use for routine diagnostic applications is less obvious and is fraught with difficulties that will be discussed below.

A more defined and targeted array is one designed for a specific region(s) of the genome for the purpose of evaluating that targeted segment. It may be designed to study a specific chromosome [10,11] or chromosomal segment[12–16] or to identify and evaluate specific DNA dosage abnormalities in individuals with suspected microdeletion syndromes[3] or subtelomeric rearrangements.[17] The crucial goal of a targeted microarray in medical practice is to provide clinically useful results for diagnosis, genetic counseling, prognosis, and clinical management of unbalanced cytogenetic abnormalities. Thus, a

well-designed array for use in a diagnostic setting should provide distinct advantages over conventional cytogenetic analysis in detecting both the majority of microscopic and submicroscopic chromosomal abnormalities, the latter of which may be missed by routine cytogenetics.

Principles of Array CGH

Array CGH is based on the same principle as traditional metaphase CGH. In both techniques, whole genomic DNA from a control (or reference) and genomic DNA from a test (or patient) are differentially labeled with two different fluorophores and used as probes that are cohybridized competitively onto nucleic acid targets. In traditional metaphase CGH, the target is a reference metaphase spread. In array CGH, these targets can be oligonucleotides, cDNAs, or genomic fragments that are cloned in a variety of vectors such as plasmids, cosmids, BACs, or P1 artificial chromosomes. In this review, we will restrict our discussion to array CGH that uses BACs as hybridization targets because oligonucleotide arrays and cDNA arrays are not currently used in clinical diagnostics. The resolution of array CGH is defined by two main factors: 1) the size of the nucleic acid targets and 2) the density of coverage over the genome; the smaller the size of the nucleic acid targets and the more contiguous the targets on the native chromosome, the higher the resolution of the array. Furthermore, a comparison of ratios between overlapping clones can narrow the region of copy-number change to within a fraction of a clone length because the fluorescence ratio for each clone represents the average copy-number ratio over the length of the entire clone.[18] The sensitivity and quantitative potential of array CGH for gene dosage measurements has been reviewed, and the usefulness of this technique in identifying gene copy number abnormalities associated with cancer has been demonstrated.[19]

CGH arrays that use large-insert genomic clones (such as BACs and P1 artificial chromosomes) are able to detect single-copy changes (ratios of 1:2 and 3:2) accurately and reliably. The use of BACs with known map positions allows direct correlation of DNA copy-number gains and losses with specific genomic sequence of known chromosomal locations.[19,20] Illustrating the flexibility afforded by this new platform are arrays that have been designed to investigate DNA copy-number changes in individual chromosomes or chromosomal regions, including chromosomes 1, 15, 18, 20, 22, and the X chromosome.[10,12–14,21] In many of these studies, array CGH identified abnormalities that were undetected by either conventional chromosome analysis or FISH.

Research Applications of Array CGH

The use of array CGH in research has accelerated the pace of gene discovery in human genetics, deepened the understanding of genomic changes in cancer, and furthered the study of fundamental concepts related to chromosome conformation, DNA methylation, histone acetylation, gene silencing, replication timing, and many other basic mechanisms pertaining to DNA structure and function.[22–26]

The high resolution afforded by array CGH has been used to define candidate regions for putative genes responsible for human genetic diseases. For example, Vissers et al[9] hybridized cell lines from two individuals with CHARGE syndrome onto a genome-wide array with a 1-Mb resolution. The authors used a 918-BAC tiling resolution array to narrow a candidate region for CHARGE syndrome on 8q12 based on data from two individuals, one with a ~5-Mb deletion and another with a more complex rearrangement comprising two deletions that overlapped that of the first deletion subject. These results allowed the authors to focus on only nine genes in the region and detect heterozygous mutations in the gene *CHD7*, which was eventually shown to be the gene for CHARGE syndrome.[9] The high resolution of that array was crucial in refining the critical region for this disease and in reducing the number of candidate genes to be investigated further.

Array CGH has proven useful in providing DNA copy number "signatures" or profiles for various cancers. Many cancers are associated with multiple gains and losses of chromosomes and chromosomal segments. Given the difficulties associated with culturing and obtaining quality metaphases from most solid tumors, approaches that directly examine the DNA content and link any dosage changes to chromosome abnormalities are highly desired. The hope of these studies is that certain signatures become prognostic markers and can guide clinical treatments. Array CGH has been applied to a large number of cancer studies with reproducible results.[27]

Diagnostic Applications of Array CGH

Few studies have been aimed at assessing the diagnostic capabilities of array CGH. Recently, de Vries et al studied 100 individuals with unexplained mental retardation.[28] All had normal GTG-banded chromosomes, and all were screened by subtelomeric multiplex ligation-dependent probe amplification with normal results. Array CGH with a tiling-resolution genome-wide microarray containing 32,447 BACs identified *de novo* alterations that were considered to be clinically relevant in 10% of the study subjects. The authors concluded that the diagnostic yield of this approach in the general population of patients with mental retardation is at least twice as high as that of standard GTG-banded karyotyping.[28] However, it is worth noting that DNA copy-number changes were identified in 97% of these patients. The majority of these alterations were inherited from phenotypically normal parents, reflecting normal large-scale copy-number variation rather than disease-associated genomic changes.

High numbers of apparently normal large-scale copy-number variation are present in all individuals.[27,29,30] Thus, whole genome arrays are likely to generate data that are difficult to interpret in a diagnostic setting; it

would be very cumbersome and expensive to evaluate one or both parents on at least 97% of cases submitted for routine studies. Such reflex testing on the parents would place an undue burden on the laboratory, resulting in unjustifiable expenses and impose unnecessary anxiety on the parents and patients. Thus, the genome-wide dense arrays that are currently available for research are not appropriate to use in a clinical diagnostic setting as these arrays raise a number of medical, technical, and financial concerns, which are beyond the scope of this review. Thus, a more targeted approach to the investigation of individuals with suspected chromosomal abnormalities would be more appropriate.

Targeted microarrays specifically designed to detect unbalanced rearrangements of the subtelomeric regions and other clinically significant regions have been constructed. Schaeffer at al. used arrays containing genomic clones for every telomere and clones for all of the microdeletion syndromes and additional selected loci spanning the genome to study 41 products of conception, which were previously analyzed by G-banding.[31] They detected all abnormalities as reported by the previously completed G-banded analysis and, in addition, discovered novel abnormalities in 4/41 (9.8%) cases.[31] More recently, we have developed and validated a microarray for the clinical diagnosis of medically significant and relatively common chromosomal alterations.[3] The chromosomal locations to be tested by the array were chosen carefully based on their clinical significance and associated known phenotypes. These and other studies[8,30] set the stage for the use of array CGH in the clinical diagnostic laboratory.

Recently, we reported our experience in 1500 consecutive cases that were submitted to our laboratory for array evaluation.[30] Our targeted array detected genomic abnormalities in ~9% of patients. Specifically, of the 1500 cases referred to our laboratory for a multitude of problems that included developmental delay, dysmorphic features, and a variety of birth defects: 134 (8.9%) showed a genomic abnormality, 36 (2.4%) showed polymorphisms or familial variants, 14 (0.9%) showed alterations of unknown clinical significance, and 84 (5.6%) showed clinically relevant genomic alterations (Figure 2). These included subtelomeric deletions and unbalanced rearrangements, microdeletions and reciprocal duplications, rare abnormalities, and low-level trisomy mosaicism. This study was not designed to be a controlled ascertainment of subjects with specific selection criteria but rather to reflect the reality of clinical cytogenetics practice. Thus, these results should provide an accurate estimate of the cytogenetic abnormalities that can be identified with a targeted microarray in a diagnostic setting. Our results showed that microarray analysis likely doubles the yield of chromosome abnormalities that is currently detected by conventional cytogenetic analysis. We should note that the array that we used was targeted to areas of the genome with known clinical significance and consisted of 832 BACs that represent only 140 loci. This was not a "whole-genome" array with consistent coverage across the genome. Therefore, in a clinical setting, a significant percentage of clinically relevant chromosomal abnormalities can be detected by judicious coverage of the genome.

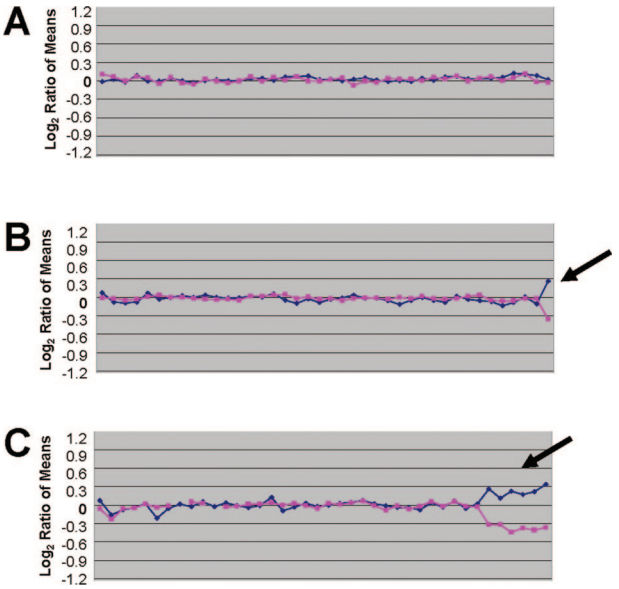

Figure 2. Array CGH for chromosome 10 in three subjects. For each panel representing chromosome 10, each clone on the plot is arranged along the *x* axis according to its location on the chromosome with the most distal, telomeric short arm clones on the left and the most distal, telomeric long-arm clones on the right. The dark blue line represents the control/patient fluorescence intensity ratios for each clone, whereas the pink line represents the fluorescence intensity ratios obtained from a second hybridization in which the dyes have been reversed (patient/control). For a deletion, the blue line deviates up, and the pink line deviates down. For duplication (not shown), the pink line would deviate up, and the blue line would deviate down. For deletion, the deviation from zero is a ratio of 1:2 (patient/control). **A:** The plot for a normal chromosome 10. Note that all $\log_2$ ratios for both experiments are zero. **B:** Terminal deletion of a single BAC clone at 10q26.3 (**arrow**). This deletion was demonstrated in a child and his clinically normal father, demonstrating a population/familial variant. **C:** Terminal deletion of six BAC clones at 10q26.3 (**arrow**). This deletion was *de novo* in origin and demonstrates the usefulness of multiple, contiguous clones from a genetic locus in providing clinical confidence in the laboratory results. In both cases (**B** and **C**), FISH confirmed the deletions. The single BAC deletion in (**B**) was familial and most likely of no clinical significance because it was inherited from a phenotypically normal parent. This illustrates our experience with most cases in which alteration of a single BAC clone at the telomere is often found in one of the parents and is therefore likely not to be clinically significant.

The use of array CGH in the clinical setting poses a unique set of challenges. The careful and prudent approach to diagnostic applications stands in sharp contrast to the more explorative world of research. In general, arrays built for research purposes are designed to screen specific chromosomal segments or the whole genome for DNA gains or losses at a very high resolution. However, microarrays constructed for diagnostic use should consider the following. First, the clones used to construct BAC arrays are usually gathered from databases and/or acquired from various academic and/or commercial sources. These should be subjected to independent FISH verification of the exact genomic location and identity of the BACs because these databases rarely provide information about the possibility that some of these BACs may map to several locations in the genome—and a substantial number map to the wrong locations.[3] Second, loci should be represented by more than one BAC clone.[3] Loci covered by only a single clone may show

dosage variation because of the intrinsic technical variability of the procedure or because of polymorphic repetitive sequences inherent to the specific locus. The use of multiple clones provides confidence in the results (Figure 2). Third, the high frequency of seemingly normal (polymorphic) large-scale copy-number variation in the human genome complicates a diagnostic analysis. These polymorphic clones should be identified and either discarded from the microarray or recognized by the laboratory before clinical use. The direct adoption of any microarray without careful consideration of clinical diagnostic use is ill advised. Such clinical use may lead to false positive diagnoses that necessitate extensive and expensive follow-up confirmatory tests by FISH or other methods; additional blood draws from unaffected relatives to determine the segregation of these deletions, duplications, or polymorphisms; and unnecessary anxiety for the families and the clinicians. A diagnostically useful microarray must be reliable, must accurately detect the chromosome abnormalities being assayed, and must provide interpretable results. Because array CGH is essentially a simultaneous FISH experiment using hundreds of clones, the costs involved are substantially reduced compared with individual FISH experiments. The only limitations to BAC array CGH are that regions not represented on an array are not assayed, smaller deletions or duplications will not be identified because this tool only interrogates gains and losses of chromatin approximating the size of a BAC clone (80 to 200 kb) or larger, point mutations will not be uncovered, and balanced chromosomal rearrangements (reciprocal translocations, Robertsonian translocations, and inversions) cannot be detected. However, even with these recognized constraints, array CGH has the potential to identify twice as many chromosome abnormalities as G-banded karyotyping.

Conclusion

Array CGH has many research applications including cancer profiling, gene discovery, and understanding epigenetic modifications and chromatin conformation. The results from such investigations can be directly correlated to genomic locations and gene expression. Thus, as a research tool, array CGH is just beginning to demonstrate its potential.

For diagnostic applications, array CGH should be approached from a different perspective. Because each clinical sample should not be viewed as a research project, diagnostic arrays should be constructed in a manner that maximizes diagnostic capabilities while minimizing false positive results to provide clinicians with diagnoses and the information that they need to manage the clinical care of individuals with identified chromosome abnormalities.

BAC arrays constructed with known clinical loci, redundancy over each region, and minimal polymorphisms provide the greatest clinical utility. Chromosome rearrangements demonstrated through array CGH can be confirmed by FISH with the same BACs demonstrating the dosage alterations. The alternative to array CGH—multiple FISH experiments—is prohibitive in cost and resources. Thus, array CGH, with its potential to identify most unbalanced microscopic and submicroscopic rearrangements, is likely to be the first approach to cytogenetic testing and will replace most banded chromosome and FISH analyses in the clinical laboratory in the near future.

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